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13. ABSTRACT *(Maximum 200*

This work describes the development of a novel system for efficient and rapid introduction of genes into specific tissues or cells in mice. Detailed analysis of gene function often relies upon introduction of genes into an animal model system. We are developing a retroviral-based system in which controlled expression of the cellular receptor for the virus in a defined pattern determines the site(s) of infection. Using this retroviral gene delivery system, a gene can be introduced not only in a spatial-specific pattern but also in a temporally-controlled manner using a retroviral vector. For these studies we expressed the receptor for the avian virus Rous sarcoma virus in mammary cells in transgenic mice using a MMTV LTR. It appears that mammary cells are infected by RSV vectors in these transgenic animals. The ability to selectively target retroviral infection has numerous applications in areas as diverse as developmental biology, gene therapy, and oncogenesis. Here we will attempt to utilize this system for targeted expression of genes in mammary cells to rapidly assess the potential of these genes to induce mammary tumors in an animal model.

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[signature] 11/10/91

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INTRODUCTION

Presently, the most common way to analyze gene function in a particular cell type *in vivo* is to generate a new transgenic line for each gene under study - a costly and time consuming endeavor. Here we describe an approach which utilizes mice expressing a retroviral receptor transgene (the Rous sarcoma virus receptor) to target infection of retroviral vectors *in vivo*. This approach allows directed infection, and thus directed gene expression, of cells expressing the viral receptor and may provide a rapid and efficient method to test the mammary tumorigenic potential of genes in an animal model. An important difference between this approach and the more traditional method of testing gene function in transgenic mice is that infection, and thus gene expression, can be temporally controlled. This allows assessment of differences in oncogenic potential of genes at different stages of mammary gland development. In addition, by allowing the gene to be introduced after gland development has occurred, the proposed strategy avoids possible effects of the intorduced genes on early gland development and might therefore provide a more relevant model of tumorigenesis. Finally, it should be possible to introduce multiple oncogenes by co-infection thereby allowing questions of synergy between these genes to be addressed.

The goals of this research project as defined by the Statement of Work are:

TASK 1, Generate transgenic mice expressing RSV receptor in specific tissues.
- a. construct MMTV LTR promoter-RSV receptor transgene.
- b. test expression of MMTV transgene in C57 mammary epithelial cells in culture. Test for susceptibility to RSV vectors.
- c. produce transgenic mice and establish lines with MMTV LTR-RSV receptor transgene

TASK 2, Characterize expression of transgene in mammary gland and other tissues.
- a. RNase protection analysis of receptor transcripts in tissues of transgenics
- b. Western blot analysis of receptor protein expression in tissues of transgenics

TASK 3, Infect transgenic animals with RSV vectors.
- a. develop protocol for infection which gives maximal infection of mammary cells (test route of infection, timing with respect to age, pregnancy etc.)
- b. produce RSV vectors carrying histochemical marker genes
- c. infect mice with vectors carrying markers (alkaline phosphatase and β-galactosidase). Score for infection by histochemical staining.
- d. infect mice with int-1 and v-myc RSV vectors and analyze tumors.

In preparation for the project outlined above, we collaborated with Steve Hughes at NCI to produced mice carrying RSV receptor transgenes. Using a muscle specific α-actin promoter/tva construct, we established five mouse lines carrying this transgene. Characterization of these lines by Western blot analysis demonstrated that the receptor was specifically expressed in several types of muscle. Although the level and pattern of expression in muscle vary for each of the transgenic lines, these experiments demonstrate that in general, the receptor can efficiently expressed without deleterious effects. Furthermore, using mice carrying a ß-actin promoter/tva construct Dr. Hughes' lab has demonstrated expression of Tva in numerous cell types including early embryonic cells. Together the results with these two different promoters suggested that expression of Tva in many different tissues and at numerous developmental periods was not detrimental. Therefore, we felt it was reasonable to attempt making transgenic animals expressing Tva in the mammary gland.

In published experiments using the α-actin-tva transgenic mice we directly demonstrated targeted retroviral infection *in vivo* [1]. 2000-5000 infectious units of an RSV vector (RCAS) carrying the bacterial alkaline phosphatase (BAP) gene (RCAS(A)-BAP) were injected into the thigh muscle of 5 day old mice. At d5 post-birth there is significant myogenesis occurring such that the myoblasts, if susceptible to RSV-A, should be good targets for infection. Controls for the experiments included injection of a subgroup E RCAS-BAP vector which should not utilize the subgroup A receptor and injection of non transgenic littermates. As expected, infection was seen only when the subgroup A virus was injected into transgenic mice. Several hundred infected myoblasts or myotubes are spread throughout the muscle and infection did not seem to be localized at the injection site. Furthermore, by injecting avian cells expressing the RSV vectors rather than the virus stock, infection of the myoblasts was dramatically increased such that thousands of cells appear to be infected. These experiments provide proof of principal for the use of Tva to efficiently target cells for RSV-A infection *in vivo.*

BODY

Last year we reported that we had made progress in several areas of the project including: a) demonstrating that a cultured mammary epithelial cell line becomes susceptible to RSV infection upon expression of Tva (task 1), b) construction of the MMTV-tva transgene construct and establishment of two lines carrying this transgene (task 2), and c) development of a protocol for production of high titer stocks of viruses carrying the RSV envelope glycoprotein (task 3). Here we report on our efforts to move the MMTV-tva transgene into a genetic background that will make the mice useful for studies on mammary tumorigenesis, on production of pseudotyped viruses carrying the RSV viral envelope, on efforts to characterize Tva expression in the transgenic mice, and finally on preliminary data using the viruses carrying RSV envelope to infect mammary cells *in vivo.* We have divided the report into sections based on the tasks described in the Statement of Work.

Task 1 Transgenic mice

MMTV LTR constructs have been used extensively to construct transgenic mice for the purpose of expressing genes in the mammary gland [2-6]. Abundant expression in mammary epithelial cells is seen when this promoter is utilized. In addition to mammary gland expression, the MMTV LTR also promotes relatively high levels of expression of transgenes in the salivary gland and the testis.

We used a construct with the MMTV LTR in the same orientation and immediately upstream of the tva coding region to generate the transgenic mice (Figure 1a). Based on previous studies using an identical arrangement of the MMTV LTR and other transgenes, this construct should promote high levels of receptor expression in mammary epithelial cells. After pronuclear injection of the MMTV LTR-tva construct and Southern blot screening of offspring, three potential founder mice carrying the transgene were identified (two male and one female). These mice were bred and offspring were screened for the transgene by Southern blotting. Figure 1b shows a representative blot analyzing DNA isolated from tails of potential transgenic mice. The Pst I digested genomic DNA shows a characteristic fragment of 365 base pairs when hybridized with a labeled probe specific for the tva sequence (marked with arrow in Fig. 1B). From the three potential founders described above, two lines of mice carrying the MMTV-tva gene have been established. These lines are named for the founder mice and are called lines #1 and #22. Thus, task 1 has been accomplished.

Unfortunately, one of the parental lines of the hybrid mouse line that is used to produce transgenic mice at the University of Pennsylvania transgenic facility has a high spontaneous mammary tumor rate. In contrast, the C57BL/6 line of mice has a low spontaneous mammary tumor incidence (less than 2%). Therefore, in preparation for use of these mice in tumorigenesis studies, we began the process of crossing our two transgenic lines with C57BL/6 mice. We have crossed the transgenic mice with C57BL/6 for 3 generations in order to move the MMTV LTR-tva transgene into the C576 genetic background where the spontaneous mammary tumor rate is low. We will continue crossing back into the C57BL/6 background for 5 generations. This will produce mice in which greater than 95% of the genetic contribution is from the low mammary tumor incidence line C57BL/6. After completing the backcross, mice homozygous for the MMTV-tva transgene will be produced (assuming the transgene does not disrupt an essential gene in either of the MMTV-tva lines). Producing homozygotes will simplify maintenance of the lines since it will obviate the need to screen mice for the transgene. In addition, this will simplify the expression and infection experiments since all the mice will carry the transgene.

I should note that moving MMTV-tva into the C57BL/6 background and all our other mouse studies have been greatly hampered this year by an outbreak of mouse hepatitis virus (MHV) throughout the University of Pennsylvania animal care facility where our mice are housed. We lost many of our transgenic animals, and more importantly we have been prevented from breeding our remaining animals while the outbreak is being eliminated. In spite of this, we have completed three generations of

backcrossing into the C57BL/6 genetic background. In addition, we are beginning to rebuild our colony of MMTV-tva transgenic animals for the infection and expression studies outlined below.

Task 2 Characterization of transgene expression

Previous studies characterizing the expression pattern of the MMTV LTR suggest that the MMTV-tva transgene should be expressed mainly in the mammary epithelial cells of the mammary gland. In addition, we would expect that the MMTV LTR will be expressed in the salivary gland and testis. To determine if the transgene is appropriately regulated in the two lines of MMTV-tva mice (described above) we proposed to look at RNA and protein expression.

Expression of Tva in the α-actin-tva transgenic mice was readily detected by both analysis of messenger RNA using RNase protection and also by western blot analysis of protein from muscle tissue using an anti-Tva antibody [1]. We have begun our studies on tva expression in the MMTV-tva mice by attempting to detect Tva protein. Mammary glands (glands # 3 and 4) from transgenic and non-transgenic lactating females were isolated and frozen (-80) till used. To prepare protein from the glands, they were homogenized in a cell lysis buffer containing SDS. The conditions used were identical to those we previously used successfully for analysis of Tva protein from the actin promoter-tva mice [1]. In addition, we also used a cell lysis protocol employing the detergent Triton X-100 instead of SDS. Protein in the Triton lysates was either analyzed directly or precipitated with the lectin concanavalin A agarose (ConA) to concentrate glycoproteins before analysis (unpublished data from our lab demonstrates that Tva is heavily glycosylated and efficiently precipitates with ConA-agarose beads).

Proteins isolated from mammary glands by SDS lysis, Triton lysis or Triton lysis and Con A precipitation were analyzed by SDS/PAGE and western blotting using a polyclonal antibody specific for Tva. To date, we have been unable to detect Tva protein in the mammary glands from MMTV-tva mice. However, these experiments have been performed with only three mice representing one of the transgenic lines (line #22). Because so few mice have been analyzed we feel it is premature to conclude whether Tva is expressed in these cells or not. As noted above, the MHV infection in our animal facility have hindered production of transgenic mice and has severely limited the number of mice available for analysis of Tva expression. Therefore, as more mice become available we will continue the protein expression analysis using mice of different ages and at different stages of pregnancy (where we expect the MMTV LTR to be actively transcribed) and also start the RNA analysis described in the original proposal.

In addition, we have purified the polyclonal anti-Tva antiserum that is used to detect the receptor protein in the transgenic tissues. A fusion protein consisting of maltose binding protein fused in frame to Tva (MBP-Tva) was produced and purified from E. coli. This protein was coupled to sepharose beads and used to purify a rabbit polyclonal anti-Tva antiserum. This purified antibody is highly reactive for Tva and has very low background on western blots or in immunoflourescence (data not

shown). When more mice are available we will use these purified antibodies to examine Tva expression in both histologic sections of mammary gland by immunofluorescence and in lysates from mammary glands of transgenic animals.

Task 3 *in vivo* infection of mammary epithelial cells

A. Virus vectors and stocks

Many viruses will acquire the envelope protein of another virus through a process known as pseudotyping. The pseudotyped virus acquires the target cell specificity of the foreign viral glycoproteins. We have taken advantage of pseudotyping to generate murine leukemia retroviruses carrying the RSV envelope glycoproteins (called MLV(RSV) psuedotypes). These pseudotypes have been produced using a transient transfection protocol similar to one previously utilized to generate high titer MLV psuedotypes with other viral glycoproteins [7-10]. Using human 293T cells and a transient transfection protocol we routinely obtain titers of MLV(RSV) psuedotypes of roughly $5X10^5$ infectious units/ml (Table 1). We have also utilized a virus concentration protocol for RSV-A which allows 100-fold concentration of virus stock [11, 12]. We have constructed vectors which express the histochemical markers alkaline phosphatase or β-galactosidase for use in these studies. This transient virus expression system allows rapid production of high titer MLV(RSV) vector stocks for use in the infection studies. Thus, we are now ready to examine the capacity of Tva to direct *in vivo* targeting of mammary epithelial cells.

B. Infection of mammary gland cells *in vivo*

The overall goal of this project is to ascertain whether Tva can efficiently direct infection of mammary epithelial cells in mice. To begin addressing this goal, we have attempted to determine if viruses carrying the RSV envelope glycoprotein can infect mammary cells in the MMTV-tva transgenic mice. Although we have not been able to demonstrate that the mice express Tva, we decided to proceed with the infection experiments since characterization of Tva expression in avian cells and in some mammalian cells transfected with tva constructs clearly shows that extremely low levels of the receptor can mediate efficient infection. Therefore, it is possible that we may not detect expression of the tva transgene, yet the cells in the mammary gland of the transgenic mice will be infectable by subgroup A RSV enveloped viruses.

The pilot infection experiments in the MMTV-tva transgenics were performed on female mice that were pregnant (17 days) to ensure that there would be actively replicating mammary epithelial cells since RSV vectors require mitosis for integration and expression of the provirus. Mice were injected with either virus harvested from 293T cells transiently transfected to produce virus (see above) or with the transfected 293T cells themselves. We had previously demonstrated that injection of avian cells expressing RSV vectors gave significantly better infection of muscle in the α actin-tva mice than

injection of cell-free virus [1]. In addition, in those studies it was demonstrated that the injected avian cells were rapidly cleared by the immune system (within 2-3 days). Three transgenic females (two from line #22 and one line #1) were injected with 10^5 infectious units of an MLV(RSV) virus encoding β-galactosidase or with 10^7 293T cells transfected to produce the same virus. Cells or cell-free virus was injected directly into mammary glands #3 or #4 respectively in MMTV-tva transgenic mice. In parallel experiments, viruses or cells producing viruses with the ecotropic MLV envelope were also injected into the mammary glands of C57BL/6 mice. Ten days after injection the mice were sacrificed and the glands were harvested. Whole mounts of the glands were prepared and stained for β-galactosidase activity.

Infection of mammary cells with the viruses carrying the RSV EnvA glycoprotein was readily detected (see Fig 2A) in all three injected transgenic animals. The ecotropic MLV virus on the other hand did not appear to readily infect the mammary cells, but rather β-galactosidase staining was only seen in the lymph node (Fig 2B). The levels of β-galactosidase staining of the glands were roughly equivalent in the two mice from line #22 and slightly higher for the line #1 mouse (the line #1 gland infected with cell-free virus is shown in Fig 2A). In contrast to the mouse infected with ecotropic MLV enveloped virus, with RSV-A enveloped virus β-galactosidase activity was not localized at the injection site but rather appeared to be spread throughout the gland in the transgenic mice. Given the level of staining and its dispersion throughout the gland, it appears that a number of cells were infected by the MLV(RSV) virus. Alternatively, the staining in these whole mounts took several weeks to develop, thus it is possible that the blue colored product of the β-galactosidase reaction diffused throughout the tissue because of a high local activity of the enzyme. In either case, the result presented suggests that cells in the mammary gland of the transgenic mice were infected by the virus carrying RSV EnvA.

The in vivo infection results strongly suggest that mammary cells were infected for two reasons. First, it is unlikely that the β-galactosidase staining in the glands injected with 293T cells was due to residual, uncleared transfected cells since no such staining was seen in the mouse injected with the 293T cells producing ecotropic MLV virus. Thus, the ß-gal activity is most likely due to infected mouse cells in the injected gland. More compelling evidence for infection comes from the glands infected with cell-free virus. In this case the observed ß-gal activity can only be due to infection of mammary cells by the incoming virus. Since this virus carries RSV envelope and since we know from numerous experiments that RSV envelope can only direct infection of cells expressing Tva, then the infection is almost certainly due to the tva transgene. However, since the control non-transgenic mouse injected with the same virus stock was lost we cannot yet be certain of these results. However, much work remains to prove that this is the case and if so to define the target cells in the mammary gland that are infected by the MLV(RSV) virus. Thus our efforts will be focused on repeating these experiments with more mice and many more controls.

CONCLUSIONS

We have made significant progress in the second year of this proposal. Task 1 is completed and we are presently re-building our transgenic mouse colony. Two lines carrying the MMTV LTR/RSV transgene were produced last year. This year, we bred these mice with a line that has a low spontaneous mammary tumor incidence (C57BL/6) to prepare for future tumorigenesis studies. Most of our efforts this year have been focused on task 3 where we established protocols for production of high titer stocks of viruses carrying the RSV envelope. Most importantly, preliminary infection experiments have been performed using these viruses and suggest that directed infection of mammary cells by the MMTV-tva transgene is possible. From this work we can conclude that: a) The MMTV-tva transgene is not toxic in vivo since transgenic lines have been established, b) The MLV(RSV) psuedotype system allows rapid and efficient production of virus stocks carrying MLV genomes and bearing RSV envelope. This will allow us to utilize the many oncogene constructs already in MLV vectors for our future work on this project. and c) In preliminary experiments, Tva appears to be capable of directing infection of mammary cells *in vivo*.

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Table 1. Titer of RSV enveloped MLV pseudotype viruses.

	Titer on Target cells[b]	
Viral Envelope[a]	**NIH3T3**	**3T3Tva**
none	0	0
EnvA	0	3-9 X 10^5
VSV-G	7-10 X10^5	7-10 X10^5

[a] The MLV virus was produced by transient transfection of 293T cells with plasmids HIT60 (expressing MLV gag-pol) and HIT111 (MLV genomic vector carrying β-galactosidase marker gene) essentially as described (8). To produced pseudotyped virus, plasmid encoding either RSV EnvA or vesicular stomatitis virus glycoprotein (VSV-G) was co-transfected with HIT 60 and HIT111.

[b] Viral titers on NIH3T3 or NIH3T3 target cells expressing Tva were determined by infecting the target cells with dilutions of media harvested from the transiently transfected 293T cells. After an overnight infection, infected cells were grown for 48 hours then fixed and stained for β-galactosidase activity. Titer is expressed as number of β-gal positive foci per milliliter of transfected cell supernatant. Range of titer observed in several independent experiments is given.

FIGURE LEGENDS

Figure 1. Southern blot analysis of the MMTV-tva transgene in DNA of line #22 offspring. Genomic DNA (10 μg) prepared from tails of 10-15d pups was analyzed for the MMTV-tva transgene. After digestion with Pst I, separation on a 1.5% agarose gel and transfer to nylon, the blot was hybridized with a random prime labeled probe specific for tva. The arrow indicates a 365 base pair band expected for the tva transgene. The pg950 lane contains a control DNA sample from the 3T3Tva cell line stably transfected with a Tva expression vector. The numbers above each lane indicate different offspring (F2 and F3 generations) derived from the #22 founder animal.

Figure 2. In vivo infection of mammary cells with MLV(RSV) virus. (A) 10^5 infectious units of cell-free MLV(RSV) virus was injected directly into the mammary gland of a pregnant mouse (d17). Ten days after injection the mouse was sacrificed and the mammary glands were removed, mounted, fixed and stained for β-gal activity. The blue color indicates production of β-galactosidase from infected cells. (B) Another mammary gland from same mouse as in (A) infected with 10^5 infectious units of cell-free ecotropic MLV virus. Mammary gland was processed and stained as in (A). Blue staining region corresponds to the lymph node underlying the gland.

Fig. 1

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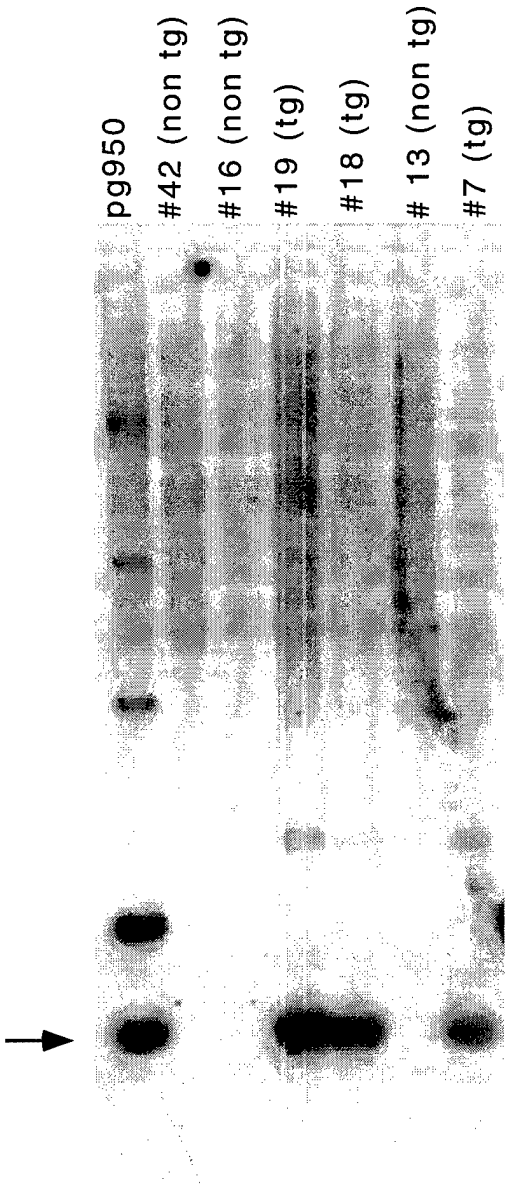

Figure 2

(A)

(B)